Supporting Information:

Unexpected Enhancement in Biological Activity of a GPCR Ligand Induced by an Oligoethylene Glycol Substituent

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I. General Methods:

All materials, unless otherwise noted, were obtained from commercial suppliers and used as provided. Anhydrous reaction solvents were distilled as follows: methanol (CH₃OH) from magnesium metal; and dichloromethane (CH₂Cl₂) from calcium hydride. Non-aqueous reactions were conducted under an inert atmosphere of nitrogen. Reactions were monitored by thin layer chromatography (TLC) unless otherwise noted. Analytical TLC was performed on EM Science TLC plate pre-coated with 0.25 mm Silica Gel 60 F₂₅₄, and compounds were visualized using UV lamp and potassium permanganate stain (3g KMnO₄, 20 g K₂CO₃, 5 mL aqueous 5% NaOH, 300 mL H₂O). Flash column chromatography was performed using silica gel 60, 230-450 mesh (Sorbent Technologies). All *N*-formyl peptide derivatives were purified by Beckman Coulter high pressure liquid chromatography (HPLC) using YMC C18 reverse phase column with 0.05% trifluoroacetic acid (TFA) in Milli-Q water as solvent A and 0.05% TFA in acetronitrile as solvent B.

¹H NMR spectra were obtained using a Varian MercuryPlus 300 (300 MHz) spectrometer, and ¹³C NMR spectra were obtained using a Varian MercuryPlus 300 (75 MHz) spectrometer. Chemical shifts are reported relative to residual solvent signals in parts per million (δ) (CDCl₃: ¹H: δ 7.27, ¹³C: δ 77.23; CD₃OD: ¹H: δ 3.31, ¹³C: δ 49.15; DMSO-d₆: ¹H: δ 2.50, ¹³C: δ 39.51). ¹H NMR data are assumed to be first order with apparent doublets and triplets reported as d and t, respectively. Multiplets are reported as m, and resonances that appear broad are designated as br. High-resolution electrospray ionization mass spectra (HRESI-MS) were obtained on a Micromass LCT.

II. Synthetic Procedures

A. Synthesis of N-formyl peptide derivatives:

Commercially available *N*-formyl-Nle-Leu-Phe **1** (0.168 g, 0.400 mmol, 1 eq.) was dissolved in dimethylformamide (DMF) (2 mL). 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate, HBTU, (0.152 g, 0.400 mmol, 1 eq.), and 1-hydroxybenzotriazole hydrate,

HOBt, (0.0540 g, 0.400 mmol, 1.0 eq.) were added to the solution. The solution of monoprotected 1-N-Boc-1,13-diamino-4,7,10-trioxatridecane (2) (0.141 g, 0.440 mmol, 1.1 eq.) and N,N-diisopropylethylamine, DIEA, (140 µl, 0.80 mmol, 2.0 eq.) in DMF (2 mL) was added dropwise (Compound 2 was prepared from commercially available 4,7,10-trioxa-1,13-tridecane diamine using a reported procedure¹). The reaction mixture was stirred for 16 h under N₂ atmosphere and concentrated under reduce pressure. Subsequently, the crude product was treated with 10% TFA in CH₂Cl₂ and 2.5% triisopropylsilane (TIS) solution (5 mL) to remove the Boc group. After stirring under nitrogen for 16 h, the reaction mixture was concentrated under reduced pressure. The resulting adduct was purified by HPLC and lyophilized to afford white powder (0.211 g, 0.293 mmol, 73% yield). ¹H NMR (500 MHz, DMSO-d₆): $\delta = 8.20$ (d, J = 8 Hz, 1 H, 8.03 (d, J = 8 Hz, 1 H), 7.98 (s, 1 H), 7.83-7.79 (m, 2 H), 7.69 (br, 2 H), 7.23-7.14(m, 5H), 4.39 (td, J = 8, 6.5 Hz, 1H), 4.28 (td, J = 8, 5.5 Hz, 1H), 4.24-4.19 (m, 1H), 3.51-3.41(m, 10H), 3.27 (td, J = 6.25, 1.5 Hz, 2H), 3.10-3.03 (m, 1H), 3.01-2.95 (m, 1H), 2.93-2.89 (m, 1H)1H), 2.82-2.78 (m, 3H), 1.75 (quint, J = 6.5 Hz, 2H), 1.62-1.16 (m, 11H), 0.84-0.77 (m, 9H); ¹³C NMR (500 MHz, DMSO-d₆): 8 174.60, 174.37, 173.48, 164.06, 140.64, 132.20, 131.12, 129.35, 72.82, 72.76, 72.62, 72.59, 70.97, 70.46, 56.95, 54.29, 54.20, 43.65, 40.83, 39.94, 38.86, 34.96, 32.24, 30.40, 30.29, 27.17, 26.08, 24.98, 24.67, 16.96; ESI-MS calcd for $C_{32}H_{55}N_5O_7$ [M+H]⁺: 622.4175, found 621.4181.

S3

¹ Fan, E.; Zhang, Z.; Minke, W. E.; Hou, Z.; Verlinde, C. L. M. J.; Hol, W. G. J. J. Am. Chem. Soc. **2000**, 122, 2663-2664.

Compound 4 was synthesized according to a reported procedure.¹ To a solution of formyl peptide derivative possessing the C-terminal substituent composed of three ethylene glycol units (11.0 mg, 17.7 µmol, 1 eq.) and triethylamine, NEt₃, (12.3 µL, 88.3 µmol, 5

eq.) in CH₃OH (1 mL), a solution of oligoethylene glycol-based linker **4** (9.12 mg, 21.2 µmol, 1.2 eq.) in CH₃OH (1 mL) was added. After stirring at room-temperature under N₂ for 16 h, the reaction mixture was concentrated under reduced pressure. The resulting product was subjected to 10% TFA in CH₂Cl₂ and 2.5% triisopropylsilane (TIS) solution (2 mL) for 6 h to liberate the amine group. The solvent was removed to give a crude product, which was purified using HPLC. Acetronitrile was removed and the resulting solution was lyophilized to afford compound **5** (10.19 mg, 11.07 µmol, 63% yield) as white powder. ¹H NMR (500 MHz, DMSO-d₆): δ = 8.20 (d, J = 8 Hz, 1H), 8.04 (d, J = 8 Hz, 1H), 7.98 (s, 1H); 7.84-7.79 (m, 2H), 7.69 (br, 2H), 7.23-7.15 (m, 5H), 4.39 (td, J = 8, 6.5 Hz, 1H), 4.28 (td, J = 8, 5.5 Hz, 1H), 4.24-4.21 (m, 1H), 3.51-3.41 (m, 28H), 3.27 (td, J = 6.25, 2.0 Hz, 2H), 3.10-3.03 (m, 1H), 3.01-2.95 (m, 1H), 2.93-2.89 (m, 1H), 2.87-2.78 (m, 3H), 1.78-1.71 (m, 6H), 1.62-1.12 (m, 11H), 0.84-0.77 (m, 9H); ¹³C NMR (500 MHz, DMSO-d₆): δ 174.59, 174.35, 173.44, 164.03, 140.64, 132.22, 131.11, 129.33, 72.85, 72.83, 72.77, 72.66, 72.65, 72.63, 72.59, 70.97, 70.54, 70.50, 70.46, 56.95, 54.28, 54.17, 43.75, 43.73, 43.67, 40.86, 39.95, 38.84, 34.99, 33.99, 32.24, 30.40, 30.28, 27.17, 26.08, 24.98, 24.67, 16.95; ESI-MS calcd for C₄₆H₇₇N₇O₁₂ [M+H]⁺: 920.5703 found 920.5669.

Compound **6** was synthesized from compound **5** using a procedure similar to that employed for compound **5**. The yield was 70%. ¹H NMR $(500 \text{ MHz}, \text{DMSO-d}_6)$: $\delta = 8.19 \text{ (d, } J = 8 \text{ Hz, } 1\text{H)}, 7.98 \text{ (s, } 1\text{H)}$; 7.84-

7.77 (m, 2H), 7.66 (br, 2H), 7.25-7.12 (m, 5H), 4.44-4.32 (m, 1H), 4.31-4.14 (m, 2H), 3.52-3.38 (m, 46H), 3.28-3.23 (m, 2H), 3.10-2.78 (m, 6H), 1.77-1.63 (m, 10H), 1.61-1.07 (m, 11H), 0.86-0.68 (m, 9H); $^{13}\mathrm{C}$ NMR (500 MHz, DMSO-d₆): δ 185.43, 174.59, 174.35, 173.44, 170.93, 164.03, 140.63, 132.21, 131.10, 129.33, 72.85, 72.83, 72.77, 72.65, 72.58, 70.96, 70.51, 70.46, 56.94, 54.27, 54.17, 43.73, 40.86, 39.96, 38.84, 34.99, 33.97, 32.23, 30.39, 30.28, 27.17, 26.07, 24.97, 24.66, 16.94; ESI-MS calcd for $\mathrm{C_{60}H_{99}N_{9}O_{17}}$ [M+H]*: 1218.7232 found 1218.7241.

$$\begin{array}{c|c} & & & & \\ & &$$

Compound **7** was synthesized from compound **6** using a procedure similar to that employed for compound **5**. The yield was 61%. ¹H NMR (500 MHz, DMSO-d₆): $\delta = 8.19$ (d, J = 8 Hz, 1 H),

8.03 (d, J = 7.5 Hz, 1H), 7.98 (s, 1H), 7.84-7.78 (m, 2H), 7.64 (br, 2H), 7.44 (br, 4H), 7.23-7.15 (m, 5H), 4.42-4.37 (m, 1H), 4.30-4.20 (m, 2H), 3.50-3.42 (m, 60H), 3.27 (t, J = 6 Hz, 2H), 3.10-3.04 (m, 1H), 3.01-2.95 (m, 1H), 2.93-2.89 (m, 1H), 2.87-2.78 (m, 3H), 1.76-1.71 (m, 14H), 1.62-1.12 (m, 11H), 0.84-0.77 (m, 9H); 13 C NMR (125 MHz, DMSO-d₆): δ 185.43, 174.59, 174.34, 173.43, 170.93, 164.02, 140.63, 132.21, 131.11, 129.33, 72.86, 72.83, 72.78, 72.65,

72.59, 70.96, 70.51, 70.47, 56.93, 54.26, 54.16, 43.75, 40.87, 39.98, 38.84, 34.99, 33.96, 32.24, 30.39, 30.26, 27.17, 26.06, 24.97, 24.67, 16.95; ESI (m/z) [M] calcd for ESI-MS calcd for $C_{74}H_{121}N_{11}O_{22}$ [M+2H+Na]⁺: 770.4361 found 770.4399.

B. Synthesis of the control compounds:

$$H_2N \xrightarrow{O}_3 \overset{O}{H} \overset{O}{H} \xrightarrow{N} \overset{O}{\longrightarrow}_3 \overset{N}{\longrightarrow} NH_2$$
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Compound **4** (20.0 mg, 46.5 µmol, 1 eq.) and monoprotected 1-N-Boc-1,13-diamino-4,7,10-trioxatridecane **2**, which was prepared from commercially available 4,7,10-trioxa-1,13-tridecane diamine, were dissolved in CH₃OH (4 mL) in the

presence of NEt₃ (33.0 μ L, 232 μ mol, 5 eq.). The reaction mixture was stirred at room-temperature overnight under N₂. The solvent was removed, and the crude product was carried on. To this crude product, 10% TFA in CH₂Cl₂ and 2.5% TIS solution (4 mL) was added to remove the Boc group. The resultant mixture was stirred at rt for 6 h under N₂. The reaction mixture was concentrated and purified using HPLC. After isolation of the relevant fractions, the acetonitrile was removed and the resulting solution was lyophilized to afford compound **8** (19.9 mg, 38.4 μ mol, 83% yield) as colorless liquid. ¹H NMR (500 MHz, DMSO-d₆): δ = 7.69 (br, 4H), 3.51-3.39 (m, 36H), 2.88-2.80 (m, 2H), 1.78-1.71 (m, 4H); ¹³C NMR (500 MHz, DMSO-d₆): δ 72.81, 72.77, 72.63, 72.57, 70.52, 70.44, 43.74, 39.91, 33.97, 30.20; ESI-MS calcd for C₂₄H₄₆N₄O₈ [M+H]⁺: 519.3389 found 519.3398.

Commercially available 1,12-diaminododecane trityl resin 10 (166 mg, 50.0 µmol) was pre-swollen by shaking in DMF (5 mL) for 10 min. The solvent was filtered, and 3,4-dimethoxy-cyclobutane-1,2-dione (316 mg, 0.250 mmol) in CH₃OH (1 mL) was added. The

reaction was rotated slowly for 16 h. After completion, the solution was removed by filtration. The resin was washed with CH₃OH (3 x 5 mL). To verify that the immobilized material

contained the desired products, a small amount of resin was exposed to 10% TFA in CH₂Cl₂, and LCMS confirmed that compound **11** was released.

To generate **12**, resin **11** was swelled in DMF (5 mL) for 10 min, and the solvent was filtered. To this pre-swollen resin **11**, 1,12-diaminododecane (0.2 g, 1 mmol) in CH₃OH (4 mL) in the presence of DIEA (87 uL, 0.50 mmol) was added. The reaction mixture was slowly rotated for 16 h, affording resin **12**. The resin was washed sequentially with CH₂Cl₂ (3 x 5 mL), CH₃OH (3 x 5 mL), and CH₂Cl₂ (3 x 5 mL). A small amount of resin was cleaved with 10% TFA in CH₂Cl₂ and characterized by LCMS to verify product formation. To resin **12**, a solution of *N*-formyl-Nle-Leu-Phe **1** (25 mg, 0.60 mmol, 1.2 eq), HBTU (23 mg, 0.60 mmol, 1.2 eq.), and HOBt (8 mg, 0.60 mmol, 1.2 eq.) in DMF (3 mL) was added in the presence of DIEA (45 uL, 0.25 mmol, 5 eq.). The reaction mixture was slowly rotated overnight. Reaction progress was monitored by Kaiser test. After apparent reaction completion, the reaction mixture was subject to filtration. The immobilized product **9** was cleaved by treatment with 10% TFA in CH₂Cl₂. The resulting material was purified using HPLC. The acetonitrile was removed, and the resulting solution was lyophilized to afford compound **9** (10.9 mg, 12.3 umol, 25 % yield). The product was verified by LC/MS and mass spectrometry. ESI-MS calcd for $C_{50}H_{85}N_7O_6$ [M+H]⁺: 880.6635 found 880.6655.

III. Material and Methods for Cell-Based Assays

Cell Culture. Human stably FPR-transfected U937 cells, obtained from E. Prossnitz (University of New Mexico), were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 10 mM HEPES (pH 7.0), and 1mg/mL active geneticin. U937 cells were purchased from ATCC. Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin.

Chemotaxis Assays. Experiments were performed using ChemoTx chambers (Neuroprobe, Inc.).² FPR-transfected U937 cells were harvested and washed in FACS buffer (3X). Different concentrations of compounds of interest (formyl peptide derivatives) were diluted in FACS buffer and placed in wells of the 96-well ChemoTx chamber. The samples were covered with the 8 μm-pore filter. Cells (16,000) were placed on top of the wells and were allowed to migrate through the filter for 2 hours at 37°C and 5% CO₂. Subsequently, non-migrating cells on the top of the filter were removed by manual scraping. Cells adhering to the filter were loosened with 2.5 mM EDTA for 30 min at 4 °C. Cells that had migrated into the bottom wells were removed, lysed, and stained with CyQUANT dye as described in the product literature (Molecular Probes).³ Fluorescence was measured at 535 nm in a plate reader and compared to a standard curve (lysed cells) to determine number of cells migrated per well. At least 3 independent experiments were performed with each repetition in triplicate. Standard error is reported.

² Boyden, S. V. *J. Exp. Med.* **1962**, *115*, 453-466.

³ Jones, L. J.; Gray, M.; Yue, S. T.; Haugland, R. P.; Singer, V. L. J. Immunol. Meth. **2001**, 254, 85-98.

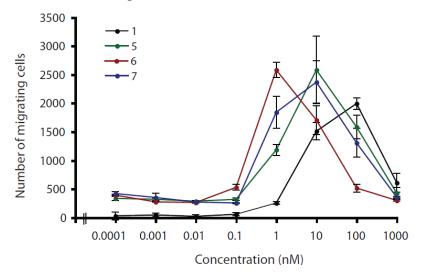
Monitoring signaling via changes in intracellular calcium ion concentration. Changes in intracellular calcium ion concentration were measured using standard methods.⁴ FPR-transfected U937 cells were washed in Hank's Buffered Salt Solution (HBSS without Ca²⁺) (3X) and resuspended in HBSS (without Ca²⁺) at 2 x 10⁶ cells/mL. Cells were loaded with 3 μM ratiometric Indo-1 AM dye (Molecular Probes) for 45 min at 37 °C and 5% CO₂. Cells were washed with FACS buffer and resuspended in FACS buffer at 2.5 x 10⁵ cells/mL. Five hundred thousand cells (2 mL) were placed in an optical glass cuvette in a Photon Technology International fluorimeter. Cells were excited at 338 nm, and the ratio of fluorescence emission was determined for Ca²⁺-bound chelator (401 nm) over unbound (480 nm). Cells were also stimulated by the ligand of interest (formyl peptide derivatives at concentration of interest), fluorescence emission ratio was measured for 5 min. The data were analyzed using the FeliX software package. The data reported include integration of the curve (area under the curve) for total increase in intracellular calcium ion concentration, and the height of the initial peak. The experiments were performed in triplicate. A similar trend was observed in each experiment.

⁴ Grynkiewicz, G.; Poenie, M.; Tsien, R.Y. J. Biol. Chem. **1985**, 260, 3440-50

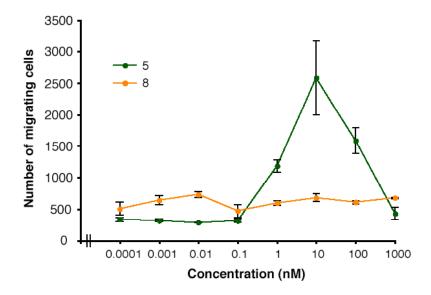
IV. Biological Data

(i) Chemotaxis Assays

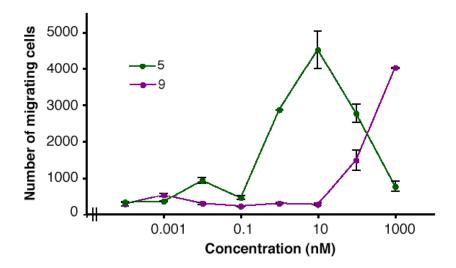
(i.i) Chemotaxis of FPR-transfected U937 cells upon stimulation with formyl peptide **1** and its derivatives (**5-7**). Data shown were derived from three separated experiments conducted in triplicate. The standard error is depicted.



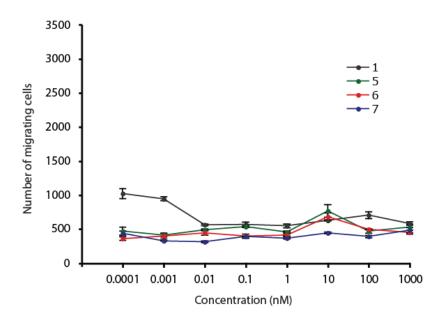
(i.ii) Responses of FPR-transfected U937 cells upon treatment with formyl peptide derivative **5** or oligethylene glycol-based linker **8**. The experiments were performed in triplicate. The standard error is depicted.



(i.iii) Responses of FPR-transfected U937 cells upon stimulation with formyl peptide derivative **5** or alkyl-based linker **9**. The experiments were performed in triplicate. The standard error is depicted.

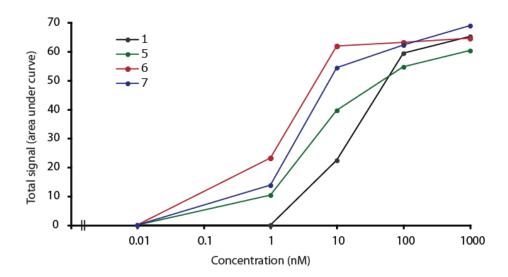


(i.iv) Chemotaxis of U937 cells upon stimulation with formyl peptide **1** and its derivatives **5-7**. The experiments were performed in triplicate. The standard error is depicted.

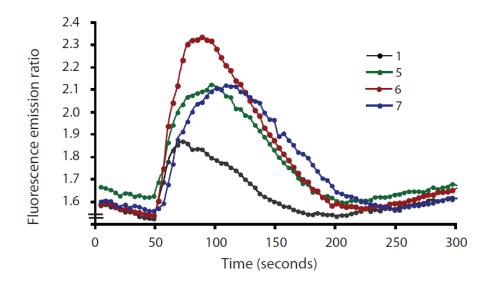


(ii) Changes in intracellular calcium ion concentrations.

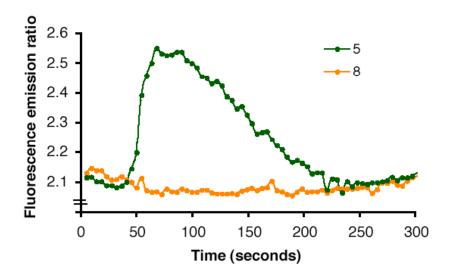
(ii.i) Total increase in intracellular calcium ions produced by formyl peptide ligands at different concentrations.



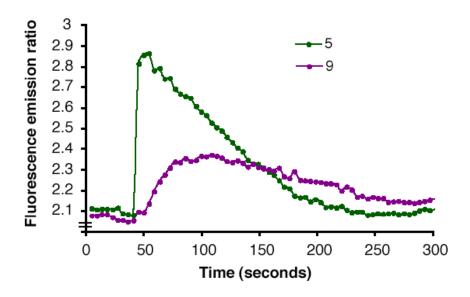
(ii.ii) Change in intracellular Ca^{2+} concentration induced upon treatment of FPR-transfected U937 with formyl peptides (10 nM).



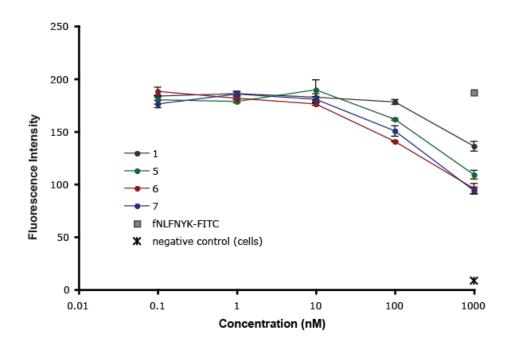
(ii.iii) Increase in intracellular Ca^{2+} concentration induced by formyl peptide **5** or oligoethylene glycol-based linker **8** at 10 nM.



(ii.iv) Increase in intracellular Ca^{2+} concentration induced by formyl peptide **5** or alkyl-based linker substituted formyl peptide **9** at 100 nM.



iii) Competitive Binding Assay.



Compound	Dissociation Constant (μM)
1	6.9 ± 0.9
5	1.5 ± 0.2
6	0.8 ± 0.08
7	0.93 ± 0.04

FPR-transfected U937 cells were loaded with 10 nM fNleLFNleYK-FITC and a compound of interest at 4 $^{\circ}$ C for 30 min. Fluorescent intensity was measured by flow cytometry.



